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Exhibit 1

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Hedgehog and *patched* gene expression in adult ocular tissues

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Abstract **We analysed the expression of members of the *hh* gene family in adult ocular tissues of newt, frog and mouse by RT-PCR method. *Shh* displayed restricted expression in the neural retina that was conserved in each species analyzed. *X-bhh*, *X-chh* and mouse *Ihh* were detected in the iris and in the retinal pigment epithelium, while mouse *Dhh* was detected additionally in the neural retina and faintly in the cornea. We also found that two types of *ptc* genes, potential *hh* targets and receptors, were expressed in these tissues, suggesting the presence of active *hh* signalling there.**

Key words: Hedgehog signalling; Adult ocular tissues; Patched genes

1. Introduction

Members of the *hh* gene family are expressed in a variety of embryonic and adult tissues, in which they are considered to function as important signalling molecules (reviewed in [1]). For example, *Shh* regulates dorso-ventral patterning of the neural tube and the somites, and the antero-posterior axis of the limb bud. *Ihh* and *Dhh* are considered to play a crucial role in the regulation of chondrogenic and testes development, respectively.

During eye development, *Drosophila hh* regulates not only the temporal assembly of photoreceptor precursor cells into ommatidial clusters in the compound eye [2–4] but also the lamina neurogenesis in the visual centers of the brain where *hh* signal from the eye is transmitted along retinal axons [5]. In vertebrates, it is considered that *hh* activity from the ventral forebrain regulates the spatial expression of *pax6*, a master control gene of eye development (reviewed in [6]), in the optic primordia, and thus regulates eye development [7,8]. Indeed, the overexpression and the defective mutant of *Sonic hedgehog*(*Shh*) result in severe eye defect [7–9] and cyclopia [7,8,10,11], respectively.

Genetic evidence in *Drosophila* indicated that *ptc*, which is a multi-pass transmembrane protein of another segment-polarity gene of *Drosophila*, is a target gene that is always induced by *hh* signal [1,12,13]. The vertebrate homologue of *ptc* has recently been cloned from mouse [14], chick [15], human [16–18] and zebrafish [19]. It has been demonstrated that they are induced by *hh* signals also in vertebrates [14,15,18]. In addition, it has been demonstrated that *ptc* is a *hh* receptor [20–22], which may regulate the spread of *hh* proteins [1,22]. In embryos, *ptc* is strongly expressed in the cells adjacent to the tissues where members of the *hh* gene family are expressed [14–19]. Furthermore, it has been observed that *ptc* RNA is transcribed in many adult tissues such as brain, lung, liver, and kidney of mouse [14] and human [18], suggesting that *hh* signalling functions not only in the embryo but also in the adult tissues.

Moreover, a recent observation suggests that *pax6* has conserved functions also in the adult eye across the vertebrates [23]. Considering the above-mentioned observation that *Shh* regulates the spatial expression of *pax6* in embryos, it is conceivable that *Shh* signalling exists in the adult eye. Indeed, it has been recently observed that *Shh* is expressed in both the ganglion cell layer and a subset of cells in the vitreal half of the inner nuclear layer in the neural retina of the adult mouse [24]. Its localization corresponds to that of *pax6* in the neural retina in zebrafish, *Xenopus*, mouse and chick [23]. In this study, we found that the expression of *Shh* in the adult neural retina was conserved in two amphibian species. In addition, our data indicated that other members of the *hh* gene family were also expressed in some adult ocular tissues in *Xenopus* and mouse, suggesting that all of the members of the *hh* gene family possess conserved functions in the adult eye across the vertebrates.

2. Materials and methods

2.1. Embryos and dissection of adult ocular tissues

Embryos of a Japanese newt, *Cynops pyrrhogaster*, and of *Xenopus laevis* were obtained by in vitro fertilization as described previously [25]. *Cynops* and *Xenopus* embryos were staged according to Okada and Ichikawa [26] and to Nieuwkoop and Faber [27], respectively. Mouse (BALB/c Cr) embryos and adults were purchased from SLC Inc. (Sizuoka, Japan). Various adult eye tissues were carefully dissected and washed in phosphate-buffered saline (PBS) under a surgical microscope, frozen in dry-ice and stored at −80°C. Between 40 and 50 eyes were used for one experiment. In both cases of *Cynops* and *Xenopus*, cells of the retinal pigment epithelium were peeled off from the choroid tissue and collected, while those in mouse were collected together with other tissues in the posterior half of the eyeball without the neural retina.

2.2. Isolation of cDNA fragments of ptc

To obtain cDNA fragments of newt and mouse *ptc* genes, the cDNA mixture prepared from the neural retina was amplified by PCR with the following degenerate primers corresponding to the conserved amino acid sequences shown in Fig. 2: ptc-U, 5′-GAGGATC-C(ACGT)(CT)T(ACGT)GA(CT)TG(CT)TT(CT) TGGGA(AG)GG-3′; ptc-D, 5′-TGGAATTCTTGGGT(ACGT)GT(ACGT)GC(ACGT)

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Abbreviations: hh, hedgehog; Shh, Sonic hedgehog; X-bhh, *Xenopus* banded hedgehog; X-chh, *Xenopus* cepharic hedgehog; Dhh, Desert hedgehog; Ihh, Indian hedgehog; ptc, patched

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GC(AG)TT(AG)AA-3'. Sequences of *ptc* genes can be accessed in GenBank (newt *ptc1*, AB000848; newt *ptc2*, AB000846; mouse *ptc2*, AB000847).

2.3. RT-PCR assays

Extraction of total RNA and RT-PCR were performed as described previously [25] with slight modifications. In some cases, the 'hot start' amplification method was employed by using AmpliTaq Gold DNA polymerase (Applied Biosystems), in which initial activation of the enzyme was carried out for 9 min at 95°C. Reactions were cycled through 1 min at 55°C, 1 min at 72°C, and 30 s at 94°C. The primers and the number of PCR cycles used for each marker are shown in Table 1. PCR reaction was carried out within the exponential phase of amplification that was judged from the radioactivity levels in the PCR products on different cycles, estimated by using a laser image analyser (Fujix BAS 2000, Fuji Film) (data not shown).

2.4. Shh and LacZ expression constructs

Synthetic mRNAs for *Shh* was synthesized by SP6 RNA polymerase from *Not* I-linearized pG3MShhΔ5'. pG3MShhΔ5'was constructed by deleting the 5' untranslated region from the *Afl* II site in the pGEM3Zf(−)(Promega)-modified plasmid vector sequence [28] to the *Bss*H II site within *Shh* cDNA [25]. The template plasmid for *Lac* Z mRNA [29] was digested with *Xho* I and transcribed by SP6 RNA polymerase.

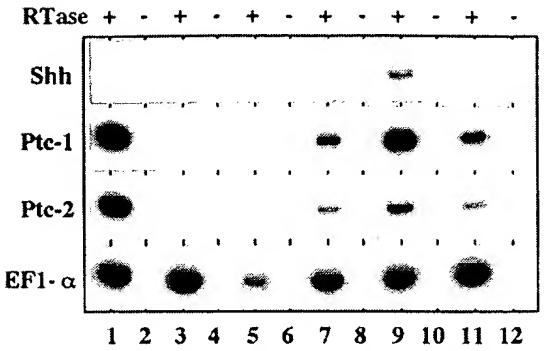

Fig. 1. Expression of *Shh* and *ptc* genes in the newt embryo and adult ocular tissues assessed by RT-PCR. Lanes 1 and 2, late neurula (st. 22) whole embryo; lanes 3 and 4, cornea; lanes 5 and 6, lens; lanes 7 and 8, iris; lanes 9 and 10, neural retina; lanes 11 and 12; retinal pigment epithelium. Total RNA (0.2 μg) from each dissected region was used to synthesize template cDNA for RT-PCR. Even-numbered lanes show negative control experiments in which reverse transcriptase was not included in the reaction of cDNA synthesis. EF1-α was used as an internal standard control.

3. Results

3.1. Expressions of Shh and ptc genes in adult ocular tissues in newt

At first, we examined the expression of *Shh* in the dissected adult ocular tissues of the Japanese newt, *Cynops pyrrhogaster*, by RT-PCR. As shown in Fig. 1, it was specifically detected in the neural retina. We considered that *ptc* should also be expressed in the neural retina if *Shh* had a function there. By using degenerate primers and the cDNA reaction mixture of the newt neural retina, we amplified and isolated two types of *ptc* cDNA fragments that were designated as *ptc1* and *ptc2* based on their similarities to mouse *ptc* genes described below. We also isolated two types of *ptc* cDNA fragments from mouse neural retina. Sequence analysis showed that one of them was identical to the *ptc* gene reported previously [14] and another was novel. In this study, we named *ptc1* for the former and *ptc2* for the novel one. Comparison of the predicted amino acid sequence of the newly isolated *ptc* homologs and of those reported elsewhere (Fig. 2) suggested that they could be grouped into at least two classes in vertebrates. RT-PCR assay demonstrated that *ptc* genes were expressed not only in the neural retina but also in other ocular tissues including the iris and the retinal pigment epithelium (Fig. 1). In addition, faint expression of *ptc1* was detected also in the cornea. Expression of both *ptc* genes was not detected in the lens.

3.2. Shh up-regulate the expression of ptc genes

To confirm that these two types of *ptc* are targets of *Shh*, we carried out the following experiment. *Cynops* embryos were injected into the animal pole of both blastomeres at the 2-cell stage with 1 ng of control *Lac* Z or *Shh* mRNAs. At the blastula stage, animal caps were isolated and cultured until the sibling control embryos reached the tail bud stage (st. 26). The expression of two *ptc* genes was assessed by RT-PCR (Fig. 3). The expression of *ptc1* was detected weakly in the control animal cap explants and was enhanced by *Shh* injection, while *ptc2* was not expressed in the control explants but was clearly induced in caps of *Shh* injection. These results indicated that both *Cynops ptc1* and *ptc2* were potential target genes in the *Shh* signalling pathway. The faint expressions of

Table 1
Primers used in RT-PCR

Animal	Gene	Forward primer	Reverse primer	Cycle no.	Ref. no.
Newt	Shh	TCTGTGATGAACCAGTGGCC	GCTACCGAGTTCTCTGCTTT	26	[25]
	ptc1	AACAAAAATTCAACCAAACCTC	TGTCTTCATTCCAGTTGATGTG	28	–
	ptc2	CACCTCTGTCGATGGCTTTA	CAGTTCCTCCTGCCAGTGCA	28	–
	EF1-α	ATCGACAAGAGAACCATCGA	GTGATCATGTTCTTGATGAA	19	[25]
Frog	X-shh	TCCGTGATGAACCAGTGGCC	GCCACTGAGTTCTCTGCTTT	26	[38]
	X-bhh	GAGAGGCACTGGCCACATTG	ATCAGCCCCACCACATTTGA	26	[38]
	X-chh*	GAACAGCTATGGTTATGATG	TGCACCTGAGTGCCATTCAC	26	[38]
	EF1-α	ATCGACAAGAGAACCATCGA	GTGATCATGTTCTTGATGAA	19	[39]
Mouse	Shh	TCTGTGATGAACCAGTGGCC	GCCACGGAGTTCTCTGCTTT	26	[40]
	Dhh	CCATCGCGGTGATGAACATG	TTATCAGCTTTGACCGATAC	26	[40]
	Ihh	CAGGTCATCGAGACTCAGGA	GGAAACAGTCGCAGGGGCCA	26	[40]
	ptc1	AACAAAAATTCAACCAAACCTC	TGTCTTCATTCCAGTTGATGTG	24	–
	ptc2	TGCCTCTCTGGAGGGCTTCC	CAGTTCCTCCTGCCAGTGCA	28	[14]
	GAPDH	GTGGCAAAGTGGAGATTGTTGCC	GATGATGACCCGTTTGGCTCC	21	[41]

*Since X-chh and X-hh4 are closely related [38], these perimers were designed for the identical sequences found in both genes.

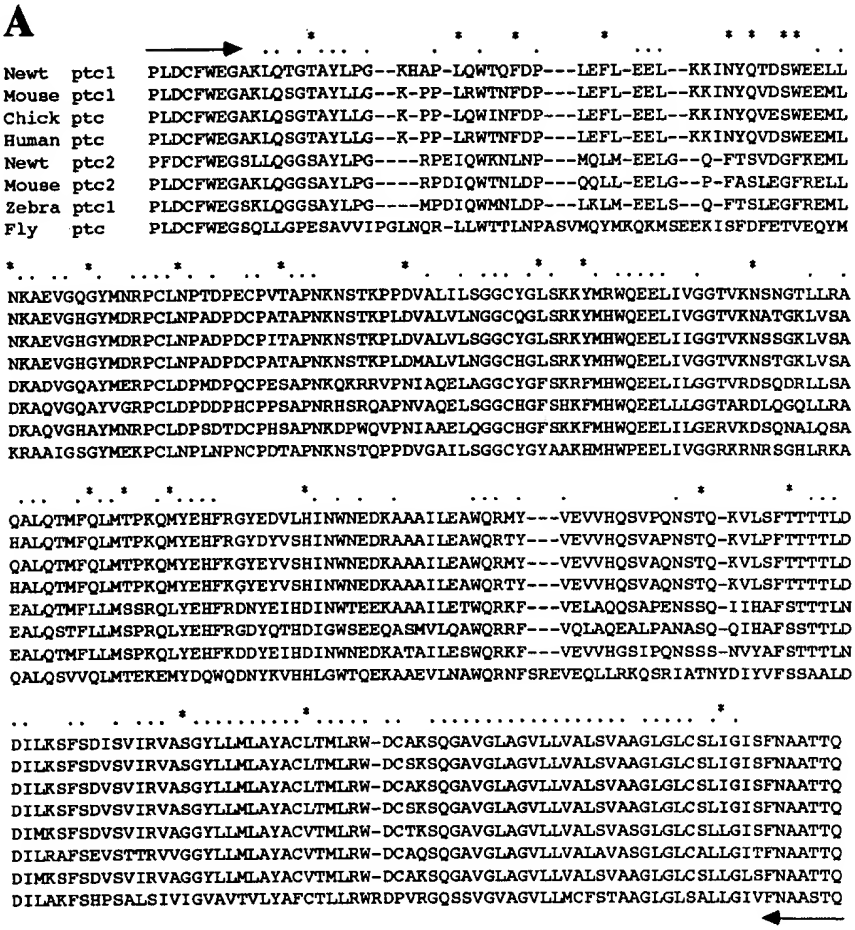

Fig. 2. A: Predicted amino acid sequences of *ptc* genes are aligned. Arrows indicate the sequences used for the design of the degenerate primers. Dots show the amino acids conserved among the vertebrate genes. Asterisks show positions in which above 3 have identical amino acid and the following 4 have another one. B: Percentage identity between the predicted amino acid sequences drawn in (A). Note that both newt and mouse *ptc2* rather than *ptc1* show higher percentage of identity to zebrafish *ptc1*.

ptc1 in the control explants suggest that *Cynops ptc1* would be expressed weakly in the epidermis. Indeed, relatively weak expression of *ptc* was observed in the embryonic rat skin [20]. In addition, the human homologue of *ptc1* is a candidate gene for the basal cell nevus syndrome, which is an inherited disorder characterized partly by predisposition to basal-cell carcinoma of the skin [16,17].

3.3. Expression of members of the hh gene family in Xenopus and mouse

To determine whether the expression of *Shh* and *ptc* in adult ocular tissues is conserved among species and why the expression of *ptc1* is extended in the cornea despite the absence of a direct connection with the neural retina, we performed similar RT-PCR experiments on other species, of

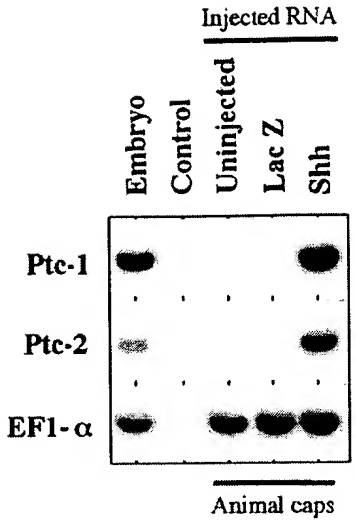

Fig. 3. *Shh* misexpression induced or enhanced the expression of *ptc1* and *ptc2* in animal cap explants. Embryos were injected in the animal pole bilaterally at the 2-cell stage with 1 ng of *Lac* Z or *Shh* RNA. At the blastula stage, animal caps were isolated and cultured until the sibling control embryos reached the tail bud stage (st. 26), and the expression of *ptc1* and *ptc2* was assessed by RT-PCR. Total RNAs from one animal cap equivalent were used as templates to generate first-strand cDNA except for the lanes marked 'Embryo' and 'Control' in which 0.2 μg of total RNA from st. 26 embryos was used. The lane marked 'Control' contains all the ingredients of 'Embryo' except for reverse transcriptase.

which *Shh* and other members of the *hh* gene family were already described.

Figs. 4 and 5 show the results in *Xenopus* and mouse, respectively. In both figures, *Shh* showed the restricted expression in the neural retina as observed in newt. Both *X-bhh* and *X-chh* in *Xenopus* and *Ihh* in mouse were detected in the iris and in the tissues containing the retinal pigment epithelium, while mouse *Dhh* was detected additionally in the neural retina and faintly in the cornea. *Ptc1* and *ptc2* in mouse displayed similar localizations to those in newt, respectively. The absence of *ptc* expression in the lens of both mouse and newt may suggest the absence of *hh* signalling there.

4. Discussion

Multiple members of the *hh* gene family have been isolated in vertebrates. Based on molecular phylogenetic analyses, they can be classified into three orthology groups: *Sonic*, *Desert* and *Indian* classes [30]. The orthologs do not necessarily have similar developmental functions in different species [30]. We previously observed that *Shh* in newt responded to basic fibroblast growth factor (FGF) differently from that of *Xenopus* in animal cap explants [25]. Since FGF signalling is likely to be involved in the regeneration processes of the limb [31–33], lens [34,35] and neural retina [36,37], in which the newt has life-long regenerative ability, we considered that *Shh* in newt might show distinctive expression in adult ocular tissues in association with the strong regenerative ability of lens or retina. However, the present data suggest the contrary; *Shh* showed restricted expression in the neural retina that was conserved in each species analyzed, rather suggesting a common function of *Shh* in the adult neural retina. In addition,

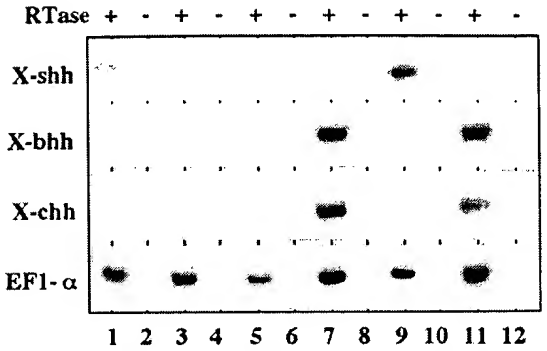

Fig. 4. Expression of *hh* genes in the *Xenopus* embryo and adult ocular tissues assessed by RT-PCR. Lanes 1 and 2, neurula (st. 15) whole embryo; lanes 3 and 4, cornea; lanes 5 and 6, lens; lanes 7 and 8, iris; lanes 9 and 10, neural retina; lanes 11 and 12, retinal pigment epithelium. Total RNA (0.2 μg) from each dissected region was used to synthesize template cDNA for RT-PCR. Even-numbered lanes show negative control experiments in which reverse transcriptase was not included in the reaction of cDNA synthesis. EF1-α was used as an internal standard control.

Jensen and Wallace [24] have recently reported that mouse *Shh* is expressed in the neural retina not only in embryos but also in adults. Our observations on *Shh* are consistent with their observation.

We found that other members of the *hh* gene family are also expressed in adult ocular tissues of *Xenopus* and mouse. Whereas *X-chh* and mouse *Dhh* genes are considered to be orthologs, they showed slightly different expression. From the observations in *Xenopus* and mouse, it is plausible that newt orthologs of *Desert* and *Indian hh* genes are also expressed in some adult ocular tissues, although we have not yet isolated them.

In this study, we isolated two types of *ptc* genes from newt

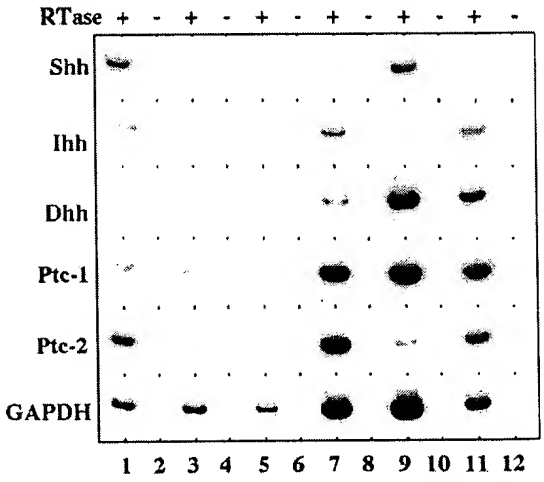

Fig. 5. Expression of *hh* and *ptc* genes in the mouse embryo and adult ocular tissues assessed by RT-PCR. Lanes 1 and 2, whole embryo (14 dpc); lanes 3 and 4, cornea; lanes 5 and 6, lens; lanes 7 and 8, iris; lanes 9 and 10, neural retina; lanes 11 and 12, posterior half in eyeball without the neural retina. Total RNA (0.2 μg) from each dissected region was used to synthesize template cDNA for RT-PCR. Even-numbered lanes show negative control experiments in which reverse transcriptase was not included in the reaction of cDNA synthesis. GAPDH was used as an internal standard control.

and mouse. The existence of two *ptc* genes in newt and mouse was unexpected since it was suggested that there is only one *ptc* gene in mouse [14], chick [15] and human [16], and that a single *ptc* is a common target of multiple *hh* signalling pathways [14,15,20]. In contrast, our data suggest that *ptc* seems to have undergone a gene duplication event at an early period during vertebrate evolution. This is consistent with the observation that there are two *ptc* genes in zebrafish [19]. Our analysis does not allow us to distinguish between the functions of the two *ptc* genes; however, it does provide the following intriguing relationship between expressions of members of *hh* and *ptc* gene families. The expression of *ptc1* but not *ptc2* in mouse was detected in the cornea where *Dhh* was expressed, suggesting the possibility that *ptc2* is not a target of the *Dhh* signalling pathway. Because human *ptc* is a tumor-suppresser gene [16,17], our findings may have significance for the study of cancer.

Although the presence of *ptc* mRNAs suggests active *hh* signalling in adult ocular tissues, the functions of all *hh* genes remain to be defined. Judging from their roles in embryos, *hh* molecules might function in certain cell–cell communications between different types of tissues in the adult eye. It should be noted, however, that recent observations suggest that *Shh* and *pax6* show similar localizations in the adult neural retina [23,24], although *Shh* downregulates the transcription of *pax6* in embryos, suggesting a difference in the role of *Shh* between the mature eye and embryos.

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